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TECHNICAL MANUSCRIPT 136

A QUANTITATIVE ASSAY
FOR CRUDE ANTHRAX TOXINS

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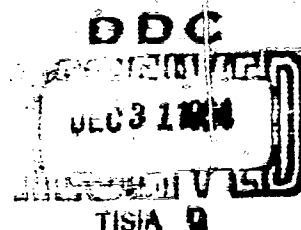
UNITED STATES ARMY
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U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 136

A QUANTITATIVE ASSAY FOR CRUDE ANTHRAX TOXINS

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ACKNOWLEDGMENT

ABSTRACT

The whole crude toxins of Bacillus anthracis, although apparently responsible for the death of animals with anthrax, had never been quantitated. A total of 14 lots of the toxic culture filtrate of B. anthracis were pooled into one large lot of crude anthrax toxins. An extensive assay of this reference material was conducted in four laboratories using the time-to-death of the IV-challenged Fischer 344 rat as the response variable. Doses of the material were varied by concentration/dilution and by volume. The data from this study were used to define a potency unit of the crude anthrax toxins. Procedures were developed and illustrated for the assay of unknown lots of the toxins by comparing the rat time-to-death response to the unknown with either (a) the responses reported in this study or (b) directly with the rat responses to a new sample of the reference toxins.

The possibilities and limitations of this standardization and of the statistical procedure through which it was developed are discussed.

I. INTRODUCTION

The excellent work of Smith, Keppie and Stanley (1955)[1] demonstrated the presence of Bacillus anthracis toxins* in the blood from guinea pigs in the terminal stages of anthrax and rekindled interest in the disease, particularly its toxins. To date, valid comparisons of results among the several experimenters[1-15] who have reported work with the toxic materials produced by B. anthracis have been difficult because either whole crude toxins or the several components have been assayed by different methods, in different assay animals, and with no reference standard of the toxins.

The paper presents the results of studies to quantitate, in terms of defined potency units, the lethality of anthrax toxins in Fischer 344 rats.** The authors have developed a reference lot of stabilized freeze-dried crude anthrax toxins. This reference material was used in the study described here and is available for other studies against which samples of anthrax toxins of unknown concentration can be assayed.

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* The toxic metabolic by-products of the growth of B. anthracis are composed of components with different biological or chemical properties. Naturally produced combinations of these components in unknown proportions will be referred to in this paper as "toxins."
** In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

II. MATERIALS AND METHODS

A. ANIMALS

Fischer 344 albino rats weighing 200-300 grams were obtained from the Fort Detrick colonies of Mr. Frank Beall and Mr. Frederick Klein. Both colonies are maintained through brother-sister matings descended from the colony described by Taylor et al.[14] This weight range was chosen because preliminary data indicated that the response time of rats that weigh more than 300 grams was significantly greater than that of rats weighing more than 200 but less than 300 grams. Further study, as reported in Table I, made on rats carefully selected for weight, revealed no significant difference within the weight range of 200-300 grams. The analysis of variance is presented in Table II.

TABLE I. RESPONSE TIME IN MINUTES OF 27 RATS INJECTED WITH 1 ML CRUDE ANTHRAX TOXINS

	Rat Weight	
200 grams	250 grams	300 grams
99	102	100
97	81	94
96	80	88
94	79	105
93	78	90
92	114	101
89	76	78
88	102	82
87	71	86
Total. 835	783	824
Harmonic Mean 92.6	84.9	90.7

TABLE II.   ANALYSIS OF VARIANCE OF RECIPROCAL RESPONSE TIMES
RECORDED IN TABLE I

Source	df.	Sum of Squares	Mean Square	F
Between weights	2	0.0485	0.0242	1.50[a]/
Within weights	24	0.3859	0.0161	
Total	26	0.4344		

a.   Nonsignificant at the 5 per cent level.

B.   RAT LETHAL TEST

Toxins of B. anthracis were injected into the dorsal vein of the penis
of the Fischer rat. In describing this test, Beall et al[11] noted a defi-
nite relationship between the dose of the toxins injected and time to death.

C.   ANTISERUM

Equine hyperimmune serum (DH-1-6C) prepared by repeated injections of
spores of the Sterne strain of B. anthracis was used.[*]

D.   PREPARATION OF ANTHRAX TOXINS

The medium described by Thorne et al[9] was made with triple-distilled
water. Subsequent to his original description, Thorne has suggested
through personal communication some changes. The contents of the medium
used in this study are shown in Table III.

1.   Media Preparation

Nine stock solutions, A, B, C, D, E, F, G, H, and I were prepared.
All stock solutions may be stored at 4°C for indefinite periods of time.

The growth medium is prepared as follows:

(a) Add 10 ml of each stock solution except that containing charcoal
to a suitable container.

(b) Add 3.6 grams of Bacto Casamino Acids (DIFCO certified).

(c) Bring the volume up to one liter with triple-distilled H_2O.

(d) Adjust pH of medium to 6.9 with 1N H_2SO_4 or 1N NaOH as needed.

(e) Dispense 460 ml of this preparation into a 3-liter Fernbach flask.

(f) Add two ml of charcoal suspension.

(g) Autoclave 20 minutes at 15 psi.

TABLE III.   CONTENTS OF STOCK SOLUTIONS USED IN THE PRESENT STUDIES

Stock Solution	Ingredient	Amount
Solution A	$CaCl_2 \cdot 2H_2O$	0.368 g/500 ml H_2O
Solution B	$MgSO_4 \cdot 7H_2O$	0.493 g/500 ml H_2O
Solution C	$MnSO_4 \cdot H_2O$	0.043 g/500 ml H_2O
Solution D[a]	adenine sulfate uracil	0.105 g 0.070 g
Solution E	thiamine HCl	0.025 g/500 ml H_2O
Solution F[b]	tryptophane cystine glycine	2.600 g 0.600 g 0.750 g
Solution G	KH_2PO_4	34.0 g/500 ml H_2O
Solution H	K_2HPO_4	43.6 g/500 ml H_2O
Solution I	charcoal (Norite A)	3.75 g/100 ml H_2O

a. Both solids were dissolved in 100 ml H_2O and the total volume was made up to 500 ml.

b. Tryptophane was dissolved in 6 ml, 6N HCl. Cystine was dissolved in 100 ml H_2O. Glycine was dissolved in 150 ml H_2O. These three solutions were combined and H_2O added to bring the total volume up to 500 ml.

2. Inoculation Procedure

Five ml of 20 per cent glucose (sterilized by filtration) was added to the Fernbach flask containing 460 ml of sterilized basal medium. Each flask of final medium was inoculated with 2 x 10⁸ Sterne strain spores. The inoculated flasks were incubated statically for 23 to 27 hours at 37°C. Four hours after inoculation, 55 ml of nine per cent NaHCO₃ was added to each flask.

This final culture was centrifuged at 3000 rpm for 30 minutes. The supernatant was decanted and 10 per cent horse serum added. The solution was then sterilized by filtration through an ultrafine glass filter.

3. Potency Testing

A preliminary test to determine the potency of each of 14 toxic filtrates was done by injecting one-milliliter samples of each filtrate intravenously into two rats. The response (death) times of the rats were considered as indications of the toxicity of each batch. The total volume per batch and the response times of the test rats are given in Table IV.

The 14 toxic filtrates were combined and a second preliminary test was conducted on the pooled material. The two rats used in this test died in 104 and 117 minutes respectively with a mean response time of 110.5 minutes. Both response times are within one standard deviation of the mean of all batches.

The pooled toxins were dispensed into 600 forty-ml drying ampules, each containing ten ml of toxins. Ampules were shell-frozen in dry ice and alcohol (-79°C). Frozen ampules were placed on an Aminco dryer* and dried under a vacuum of 10 to 30 microns of mercury for 18 to 24 hours. Ampules were cut and sealed under vacuum, packed in cardboard containers, and stored at -20°C.

In a third preliminary test, one randomly selected ampule was reconstituted with ten ml of triple-distilled water. One ml of this toxic material was assayed in each of five rats. Their mean response time was 117.2 minutes. To further test the toxicity, 0.2 ml of undiluted and of serial twofold dilutions of the reconstituted material were injected into the shaven sides of a guinea pig and observed for edematous reaction. The material reacted at a dilution of 1/32 and can be expressed according to Thorne et al[8] as containing 32 toxic units. Additional vials were reconstituted to 4X concentration and tested on immunodiffusion plates against the standard spore antiserum.[9] Three individual lines of precipitate appeared in parallel arrangement when tested with a linear pattern. The strongest precipitate line was identified as the protective antigen (Factor II) component when compared with a standard.[11] An undiluted sample of the resuspended material had a protective antigen titer of 1:64 against the standard spore antiserum.

* American Instrument Co., Silver Spring, Md.

TABLE IV. VOLUME PER BATCH AND RESPONSE TIME OF RATS
CHALLENGED WITH TOXINS BY BATCH

| Batch | Total Volume, ml | Response Times, minutes | | |
		Rat A	Rat B	Mean
1	450	97	92	94.5
2	450	107	91	99.0
3	450	97	96	96.5
4	460	95	a/	95.0
5	420	122	124	123.0
6	450	114	125	119.5
7	510	116	90	103.0
8	410	121	120	120.5
9	370	88	82	85.0
10	510	90	94	92.0
11	465	106	94	100.0
12	425	106	92	99.0
13	425	117	121	119.0
14	300	100	117	108.5
	Total 6095			Mean 103.9
				SD = 12.14

a. Missed the vein.

E. REFERENCE TOXINS

These preliminary tests constituted quality control measures on the remaining 597 vials of dried toxic filtrate. As a result of these tests it was known that these vials contained the known components of anthrax toxins.

F. PROCEDURES

The toxins were assayed independently by each of four investigators. The procedures followed by each of the four were as nearly the same as could be achieved.

The characterization of the dose-response relationship of the toxins in Fischer rats was based on an assay in which the two dose factors of amount and concentration of toxins were each tested at several levels as follows: (a) five levels of the amount of toxins designated as 4 ml, 2 ml, 1.5 ml, 1 ml, and 0.5 ml; (b) seven levels of the concentration of the toxins designated as 4X, 2X, 1X, 0.5X, 0.25X, 0.125X, and 0.0625X, where 1X is defined as the concentration resulting when one ampule is reconstituted to ten ml with a diluent of triple-distilled water. Dilutions beyond 1X were made with distilled water plus 10 per cent normal horse serum.

The 7 x 5 factorial combinations of the several levels of these two factors, plus 19 control groups, were each tested in two Fischer rats by each of four investigators as shown in Table V. Three sets of control animals are not shown in this table. The first set included five pairs of rats. Each pair was inoculated with one of the five amounts of diluent alone (i.e., triple-distilled water plus ten per cent normal horse serum), to provide assurance that their companion animals responded to toxins as opposed to the inoculation of the diluents. The second set included seven pairs of animals. Each pair in this set was inoculated with 1.5 ml of one of the seven concentration of toxins mixed with 0.5 ml (1/3 by volume) of specific antiserum.[a] The seven pairs of animals in the third set of controls were inoculated with 1.5 ml of one of the seven concentration of toxins mixed with 0.5 ml of normal horse serum. These animals provided assurance that the control No. 2 animals that lived were saved by the antiserum specific against anthrax toxins.

Each investigator required 32 ampules of dried toxins. Each of the 32 ampules was opened and reconstituted with 2.5 ml of diluent precooled to 4°C. The contents of all 32 ampules then were pooled, providing a total of 80 ml of reconstituted toxins at a concentration of 4X (4 times the original). All concentrations of toxins were maintained continuously at 4°C. To make the next dilution, 40 ml of the 4X pool were combined with 40 ml of diluent (triple-distilled water). This provided 80 ml of toxins at a concentration of 2X. Further serial twofold dilutions were made to 0.0625X (1/16X original concentration) and inoculated as planned.

TABLE V. RESPONSE TIMES IN MINUTES OF 280 FISCHER RATS
BY DOSE, CONCENTRATION, TECHNICIAN, AND RAT

		Dose, ml									
		4		2		1.5		1		0.5	
Conc.	Oper.	Rat A	B	A	B	A	B	A	B	A	B
4X	1	58	55	53	54	57	57	61	60	76	71
	2	53	61	54	52	64	63	64	63	85	70
	3	57	62	56	52	58	56	64	62	78	72
	4	60	52	448	53	59	123	63	59	81	82
2X	1	57	57	61	63	59	61	72	70	100	89
	2	57	55	65	62	74	65	84	77	119	94
	3	50	56	56	58	66	77	72	78	109	117
	4	67	56	55	65	67	88/	127	S	107	83
1X	1	53	55	70	69	119	70	90	91	127	159
	2	73	64	78	72	82	81	61	100	181	199
	3	65	62	77	80	89	83	107	97	293	483
	4	S	63	S	S	S	100	132	S	161	202
0.5X	1	70	77	153	143	129	134	145	148	S	S
	2	74	83	114	103	138	131	425	281	S	S
	3	75	69	113	118	137	151	1588	244	S	S
	4	74	94	S	139	149	S	S	400	S	S
0.25X	1	111	112	173	176	S	481	S	S	S	S
	2	136	176	295	274	S	S	S	S	S	S
	3	103	124	S	300	S	S	S	S	S	S
	4	S	118	S	S	S	S	S	S	S	S
0.125X	1	185	195	S	S	S	S	S	S	S	S
	2	253	588	S	S	S	S	S	S	S	S
	3	473	234	S	S	S	S	S	S	S	S
	4	S	S	S	S	S	S	S	S	S	S
0.0625X	1	S	S	S	S	S	S	S	S	S	S
	2	S	S	S	S	S	S	S	S	S	S
	3	S	S	S	S	S	S	S	S	S	S
	4	S	S	S	S	S	S	S	S	S	S

a. S indicates survival.

Each investigator required 108 rats. These rats were caged in 54 con-
secutively numbered cages each containing two animals. Each of the 54
treatment combinations was given to the two animals in one cage at the same
time. The random order of the treatments was specifically prescribed to
each investigator. Response times to death in minutes were recorded for
each rat and constituted the basic data.

III. RESULTS

The response times for animals are presented in Table V. Although none of the controls appears in this table, it should be noted here that none of either the first or second groups of control animals died. Some animals in the third control group challenged with 1.5 ml of toxins plus normal horse serum responded nearly the same as test animals challenged with 1.5 ml of toxins. The mean response times, in minutes, of these control animals by concentration of toxins are recorded in Table VI. The pattern of responses by the controls provides the needed assurance that the response of the test animals was specifically to the toxins of B. anthracis.

TABLE VI. MEAN RESPONSE TIME BY DOSE AND CONCENTRATIONS OF TOXINS

Concentration of Toxin	Dose, ml					Mean	Control[a]
	4	2	1.5	1	0.5		
4X	57.5	53.5	59.0	62.3	75.0	60.7	60.0
2X	55.2	60.7	66.4	75.2	105.1	69.0	70.0
1X	61.3	74.1	85.1	88.0	198.7	86.3	134.0
0.5X	74.4	121.6	136.3	247.0	s[b]	151.3	154.0
Mean	61.3	70.3	78.3	89.4	143.5	91.3	

a. 1.5 ml toxins plus normal horse serum - See text.
b. All animals survived.

In spite of carefully controlled procedures and techniques, the results from one laboratory (Technician 4) were so erratic that they were disregarded in any further analysis. Inspection of these data show that Technician 4 was the only one having reversal of results, i.e., a greater amount of toxins not killing, but lesser amounts killing, or only one of the two test animals responding (except at doses eliciting a response above 300 minutes).

The reciprocals of the response times were used for analysis because reciprocal response times are nearly normally distributed with equal variances whereas the untransformed response times are positively skewed with unequal variances.[15] The analysis of variance on the reciprocal response times of 120 rats from the four highest concentrations and the five doses is shown in Table VII. This analysis shows that both dose level and concentration have statistically significant effects on the response time of Fischer rats injected intravenously with anthrax toxins.

TABLE VII. ANALYSIS OF VARIANCE OF RECIPROCAL RESPONSE TIMES

Line No.	Effect	df.	Sum of Squares	Mean Square	F[a]
1	Dose (D)	4	11.9272	2.9818	229.37[b]
2	Concentration (C)	3	16.5629	5.5210	424.69[b]
3	Technician (T)	2	0.1543	0.0772	5.94[c]
4	D x C	12	1.7984	0.1499	11.53[b]
5	D x T	8	0.1485	0.0186	1.43
6	C x T	6	0.1180	0.0197	1.52
7	D x C x T	24	0.6452	0.0269	2.07
8	Error	60	0.7814	0.0130	
9	Total	119	32.1360		

a. Error line 8 was used to test all effects.
b. Approximate probabilities <0.001.
c. Approximate probabilities <0.05.

The analysis further shows an interaction between dose and concentration to be statistically significant. The mean response times given by dose and concentration of toxins in Table VI show that the magnitude of this interaction is slight and had no practical significance in the further analysis and interpretation of these data.

The analysis also shows a statistically significant difference among technicians. Inspection of the data shows that mean response times for all rats responding for Technician 1, 2, and 3 are respectively 78, 83, and 83 minutes. This is a practically unimportant difference, which we believe may in part be due to environmental factors since genetic differences would be almost nil after 100 generations of inbreeding. The rats used by Technician 1 came from the Beall colony, which was maintained in a different environment than the Klein colony animals used by the other two technicians. This raised the question as to the effect on this assay of Fischer rats procured from non-Detrick sources. In order to examine this effect, commercially available Fischer rats obtained from two breeders were tested and found to be suitable for this assay.

In this study, twenty Fischer 344 rats from each of two suppliers, Microbiological Associates, Inc., Bethesda, Md., and Charles River Breeding Laboratories, Inc., 1018 Beacon Street, Brookline 46, Mass., were challenged in each of two laboratories. The response times of all 80 rats are reported in Table VIII. No statistically significant difference in time of response for animals from the two suppliers was observed. A difference between the two operators and the interaction of operator with supplier was statistically significant at the five per cent level. The mean response time of three of the four groups differed by less than one minute, and the fourth group differed from the other three by approximately five minutes, a difference that could be caused by about seven units of toxins and is well within two standard errors of an estimated potency. Thus, this difference, although statistically significant because of the large number of animals tested, is considered of no consequence concerning this assay.

A test to determine the storage characteristics of the reference toxins was conducted on a vial of the toxins that had been stored for 36 months. The test vial was reconstituted with ten ml of triple-distilled water. Six rats were then challenged with these reconstituted toxins, according to the protocol described in this paper.

The estimate of potency from that test was 32.4 potency units per ml at the 1X concentration. This is essentially identical to the 32 units per ml set up in the definition. It was, therefore, concluded that the reference toxins had not changed with respect to potency during 36 months of storage.

TABLE VIII. RESPONSE TIMES IN MINUTES BY SUPPLIER, LABORATORY, AND RATS

Rat	Charles River Breeding Labs., Inc. Laboratory 1	2	Microbiological Associates, Inc. Laboratory 1	2
1	83	87	91	85
2	88	84	84	89
3	86	86	91	89
4	83	82	88	85
5	91	84	89	92
6	87	89	88	84
7	94	88	90	101
8	88	83	92	87
9	87	83	96	102
10	91	86	77	87
11	105	83	89	93
12	94	85	94	79
13	92	79	90	107
14	90	81	91	88
15	98	81	91	83
16	91	85	77	90
17	82	83	97	89
18	90	87	89	88
19	83	85	82	75
20	88	83	90	86
Harmonic Mean Response Time	89.28	84.10	88.50	88.42

IV. DEVELOPMENT OF ASSAY PROCEDURES

A. DIRECT ASSAY METHOD

A potency assay should be based on dose expressed in terms of well-defined units. No such units have as yet been defined for anthrax toxins. Varying the amount of toxins by varying either dose or concentration would have a significant effect on the response time of rats; however, rats injected with one ml of toxins concentrated to 2X responded in about the same time (75 minutes) as rats injected with two ml of toxins concentrated at 1X (74 minutes). This relationship holds true for most other dose-by-concentration combinations for which the product of these two factors is a constant. If doses are converted into 0.5-ml units and concentrations into 0.0625 units then the doses and concentrations in the margins of Table V can be expressed as shown in the margins of Table IX.

TABLE IX. DERIVATION OF POTENCY UNITS OF ANTHRAX TOXINS

Concentration of Toxins in 0.0625-Fold Units	Dose of Toxins in 0.5-ml Units				
	8	4	3	2	1
64	512	256	192	128	64
32	256	128	96	64	32
16	128	64	48	32	16
8	64	32	24	16	8
4	32	16	12	8	4
2	16	8	6	4	2
1	8	4	3	2	1

The products of the marginal numbers in Table IX for any two equivalent dose-by-concentration combinations are the same; thus, the product of 2 dose units with 32 concentration units gives 64 total potency units of toxins. Similarly, 4 dose units of 16 concentration units also contain 64 total potency units of toxins. We define the potency unit of anthrax toxins to be expressed as these products of dose by concentration of this particular lot of toxins.

If we were to carry the definition of a potency unit no further, then one ml of 1X concentration of any anthrax toxins, regardless of its actual effect in animals, would have 32 potency units. In order to standardize a potency unit it is necessary to describe the association between the dose in units and the potency in terms of a biological response to this particular lot of anthrax toxins. The potency of any other lot of toxins may then be measured by comparing the response to a known amount of the test toxins with the response to the same amount of the reference toxins.

These response characteristics are described as the dose-response relationship when measured doses of these toxins are injected intravenously into Fischer 344 rats. The challenged rats responded by dying at a time that is shown here to be highly dependent on the dose measured in potency units of these toxins.

The regression of mean reciprocal response times on the $\log_2$ of the potency units of anthrax toxins is shown in Figure 1. The least squares line has the equation:

$$Y = b_0 + b_1 X + b_2 X^2 \tag{1}$$

where Y is the mean reciprocal response time, X is the potency of anthrax toxins in $\log_2$ units and the b's are regression coefficients computed from the data of this test. The values of the coefficients, their variances and covariances are:

$b_0 = -2.591$	$V(b_0) = .077121$	$V(b_0 b_1) = -.026902$
$b_1 = 0.959$	$V(b_1) = .009514$	$V(b_0 b_2) = .002238$
$b_2 = -0.051$	$V(b_2) = .000068$	$V(b_1 b_2) = -.000800$

This regression line represents a basis upon which comparisons of potency of anthrax toxins can be made. Thus test toxins can be assayed either indirectly against this curve or directly with parallel assays of the reference toxins.
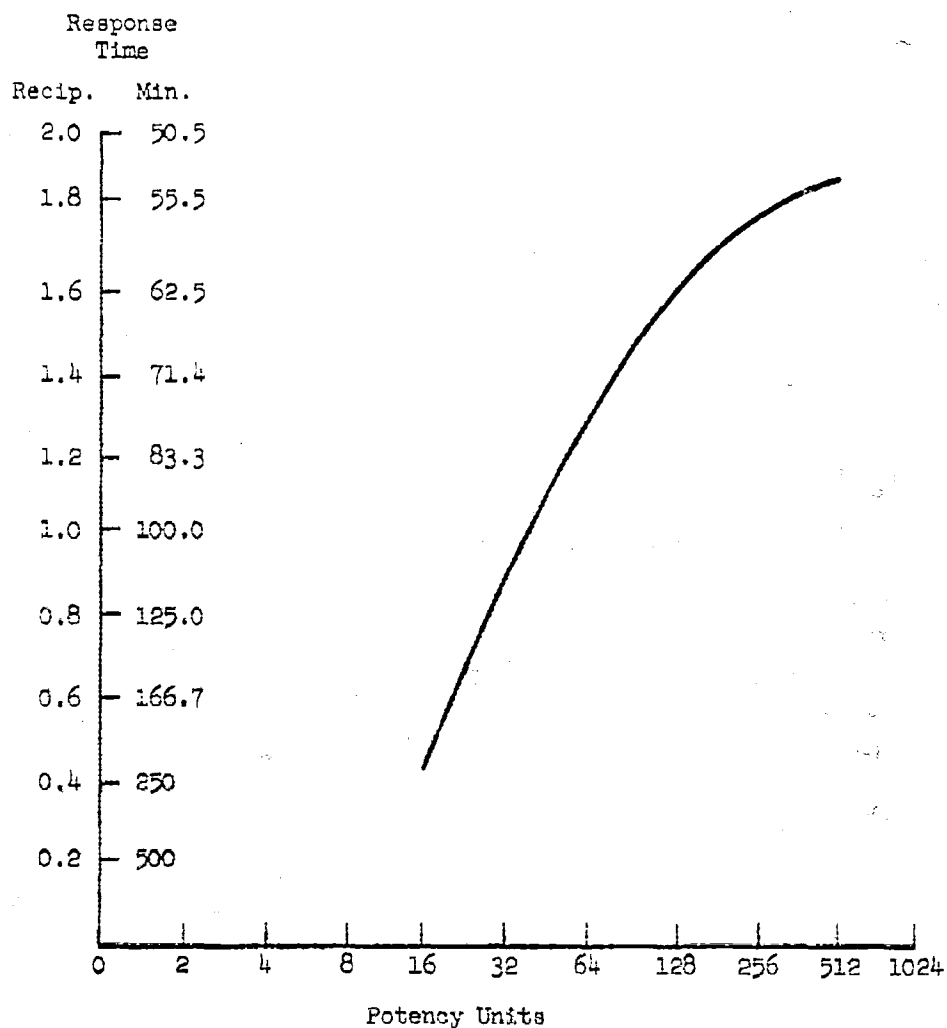
Response
Time

Recip.   Min.

2.0 ┬ 50.5

1.8 ┤ 55.5

1.6 ┤ 62.5

1.4 ┤ 71.4

1.2 ┤ 83.3

1.0 ┤ 100.0

0.8 ┤ 125.0

0.6 ┤ 166.7

0.4 ┤ 250

0.2 ┤ 500

```
    0   2   4   8   16   32   64   128   256   512   1024
```

Potency Units

Figure 1.   Regression of Reciprocal Response Time of Fischer Rats on
            Log Dose of Anthrax Toxins Expressed in Potency Units.

B.   INDIRECT ASSAY METHOD

In order to use the responses of 120 rats to the reference toxins for
which the slope of the dose response curve has been calculated, we recom-
mend the use of the indirect method of assaying unknown anthrax toxins for
potency. The regression is nearly linear for doses from 16 to 128 units,
corresponding to response times from 240 to 65 minutes. Thus, although the

concentration of test or unknown toxins is arbitrary, it should be of such concentration that one ml, injected intravenously, will kill a Fischer rat in no less than 65 minutes nor more than 240 minutes.

To test the potency of test or unknown toxins, enough animals should be used so that the amount of variation in the final result that can be attributed to the test rats is at least no greater than the amount of variation contributed by the standard rats. Thus, at least six Fischer rats of 200-300 grams from a suitable colony should be intravenously inoculated, three with two ml of the test toxins and three with one ml.

The test is based on the mean reciprocal response times of the rats.* This is simply the reciprocal times to death of the rats in minutes ($100/t$) summed up and the average calculated. The reciprocal response times of the rats can be put in the following form:

	Reference Toxins			Test Toxins	
	$Y = 100/t$			$Y = 100/t$	
	1 ml	2 ml		1 ml	2 ml
	1. ___	4. ___		1. ___	4. ___
Rat 2. ___		5. ___	Rat 2. ___		5. ___
	3. ___	6. ___		3. ___	6. ___
	ΣY ___	ΣY ___		ΣY ___	ΣY ___
$\bar{Y} = R_1$ ___		R_2 ___	$\bar{Y} = T_1$ ___		T_2 ___
$R_1 + R_2 =$ ___			$T_1 + T_2 =$ ___		

where R_1, R_2, T_1, and T_2 are mean reciprocal response times. This form for calculation can be used for either the direct or indirect assay method.

The estimate of the difference in potency (D) between the test toxins and the reference can be found as:

$$D = \frac{(T_1 + T_2) - (R_1 + R_2)}{2L} \tag{2}$$

---

* Since the rat response is very uniform any observed non-response must be considered the result of technique at some stage of the assay procedure.

where the letters T and R represent the mean reciprocal response times from the table above and L is the average slope of the reference dose-response curve at the two dose levels used in the test. This average slope may be calculated as:

$$L = b_1 + b_2 \ (X_1 + X_2) \tag{3}$$

where X_1 and X_2 are the dose levels of the reference toxins (in $\log_2$ potency units) that were used in the test, and b_1 and b_2 are the estimates of the regression coefficients from Equation (1). When the test is run using 1-ml and 2-ml doses of toxins, then $X_1 = 5$ and $X_2 = 6$. Under these conditions $R_1 = 0.92$, $R_2 = 1.34$ from (1) and $L = 0.3985$ from (3) so that Equation (2) becomes:

$$D = \frac{(T_1 + T_2) - 2.26}{0.7970} \tag{4}$$

The letter D represents the amount of difference between the test and reference toxins in terms of $\log_2$ potency units. If D is positive then the test toxins are more potent than the reference and vice versa. Since the reference toxins have a potency of 5 $\log_2$ units/ml at a concentration of 1X, the potency (P) of the test toxins in $\log_2$ units at the concentration tested will be found as:

$$P = 5 + D \tag{5}$$

To find the number of potency units per ml of the test toxins, its potency needs to be converted from $\log_2$ units to $\log_{10}$ units. The conversion formula is:

$$\log_{10} P = \log_2 P \ \log_{10} 2$$

The value of P in units is found by looking up the antilog of this product. This value will be the number of potency units per ml of the test toxins at the concentration tested.

C. ESTIMATION OF VARIANCE

There is variation inherent in this assay system in addition to the variation between samples of toxins. Thus the single estimates of the potency of any particular sample of unknown toxins should be bounded by confidence limits. In order that these limits may be determined it is necessary to calculate the variance of the estimate D of the $\log_2$ of the difference in potency between the test and the reference. The variance of the estimate D will depend on the variance of the observed response times and the regression.

If we express:

$$D = \frac{N}{G} \tag{6}$$

then

$$V(D) = \frac{1}{G^2}\left\{V(N) + D^2 V(G)\right\} \tag{7}$$

which will apply because N and G are estimated from independent observations.[15] Since the four mean reciprocal response times are stochastically independent, the estimate of $V(N)$ can be expressed as:

$$V(N) = V(R_1) + V(R_2) + V(T_1) + V(T_2) \tag{8}$$

where $V(T_1)$ and $V(T_2)$ are obtained directly from the data of the test and $V(R_1)$ and $V(R_2)$ are calculated from the regression line as:

$$V(R_1) = V(\bar{Y}) + (X_1 - \bar{X})^2 V(b_1) + (X_1^2 - \bar{X}^2)^2 V(b_2) \tag{9}$$

The variance of G is given by the equation:

$$V(G) = 4\left\{V(b_1) + (X_1 + X_2)^2 V(b_2) + (X_1 + X_2) V(b_1 b_2)\right\} \tag{10}$$

When the test is run using one-ml and two-ml doses of toxins, then $X_1 = 5$ and $X_2 = 6$. Under these conditions:

$$V(R_1) = 0.0134, \quad V(R_2) = 0.0018 \text{ and } V(G) = 0.0355$$

so that:

$$V(D) = \frac{1}{0.6352}\left\{V(N) + 0.0355D^2\right\} \tag{11}$$

and:

$$V(N) = 0.0134 + 0.0018 + V(T_1) + V(T_2) \tag{12}$$

EXAMPLE

A sample of toxins of unknown potency was tested in this laboratory. It was known to kill Fischer rats in slightly more than 90 minutes when injected intravenously in doses of one ml at a concentration of 1X. The response of the unknown toxins was compared with the response curve described by Equation (1). Each of three Fischer rats was injected with one ml of the test toxins and their reciprocal response times in minutes were recorded in the appropriate spaces on the form called Figure 2. Three other

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Reference   Toxin   Test   Toxin

Y = 100/t   Y = 100/t

| ml.   2 ml. | ml.   2 ml.

Rat $\begin{cases} 1 \\ 2 \\ 3 \end{cases}$ _____ _____   Rat $\begin{cases} 1 \\ 2 \\ 3 \end{cases}$ $\begin{matrix} 1.39 & 1.67 \\ 1.25 & 1.56 \\ 1.15 & 1.59 \end{matrix}$ $b_0 = -2.5912$
$b_1 = 0.9592$
$b_2 = -0.0510$

$\sum Y$ _____ _____ $\sum Y$ 3.79   4.82

$\bar{Y} = R_1$ 0.92   1.34 $\bar{Y} = T_1$ 1.26   1.61 $V(b_0) = 0.07712089$

$R_1 + R_2 =$ 2.26 $T_1 + T_2 =$ 2.87 $V(b_1) = 0.00901355$

$\sum Y^2$ _____ _____ $\sum Y^2$ 4.8171   7.7506 $V(b_2) = 0.00006804$

$V(R_1)$ 0.0134   0.0018 $V(T_1)$ 0.0048   0.0011 $V(b_1 b_2) = -0.000800$

$L = b_1 + b_2 (x_1 + x_2)$

$x_1 = $ 5 $x_2 = $ 6 $(x_1 + x_2) = $ 11 $(x_1 + x_2)^2 = $ 121

$b_1 = 0.9592$

$b_2(x_1 + x_2) = 0.5607$ $D = \dfrac{(T_1 + T_2) - (R_1 + R_2)}{2L} = \dfrac{2.87 - 2.26}{0.7970} = 0.78$

$L = 0.3985$

$2L = 0.7970$ $D^2 = 0.6084$

$4L^2 = 0.6352$ $\log_2 P = 5 + D = 5$

$\log_{10} P = 0.301 \times 5.78 = 1.74$ $P = \boxed{55.0 \; U/ml}$

$V(G) = 4 \left\{ V(b_1) + (x_1 + x_2)^2 \, V(b_2) + (x_1 + x_2) \, V(b_1 b_2) \right\} = $ 0.0355

$V(N) = V(R_1) + V(R_2) + V(T_1) + V(T_2) = $ 0.0211

$V(D) = \dfrac{1}{4L^2} \left\{ V(N) + D^2 \, V(G) \right\} = $ 0.0672

$SE(D) = $ 0.26

$UL(D) = $ 1.30 $\log_{10} UL(P) = $ 1.90 $UL(P) = $ 79.4

$LL(D) = $ 0.26 $\log_{10} LL(P) = $ 1.58 $LL(P) = $ 38.0

Figure 2.   Calculation Form for Anthrax Toxins Potency.

Fischer rats were each injected intravenously with two ml of the test tox-
ins. Their reciprocal response times were also recorded on Figure 2. From
these six reciprocal response times values of T_1 and T_2 were calculated.
Corresponding values of R_1 and R_2 were obtained from the regression line by
substituting respectively the values 5 and 6 for X in Equation (1). The
value of L was calculated from Equation (3) using the values 5 and 6 for X_1
and X_2. The values 5 and 6 were used in these two cases because they are
the $\log_2$ of the number of units in one ml and two ml of the reference
toxins.

The value of D was calculated by substituting the previously calculated
values of R_1, R_2, T_1, T_2, and L in Equation (2). This value of D was found
to be 0.78. This indicates that the test toxins were 0.78 $\log_2$ unit more
potent than the reference. One ml of the reference toxins contains 5 $\log_2$
units so the test toxins must contain 5.78 $\log_2$ units. The value of P in
units can readily be determined by looking up the antilog of the product of
5.78 and 0.301. This product is 1.74, the antilog of which is 55.0. Thus
the test toxins have 55.0 potency units per ml at the concentration tested.

The formulas for calculating the variance of the estimate D of the $\log_2$
of the difference in potency between the test and the reference are de-
scribed above as Equations (6) through (10). These calculations were made
in this example and it was found that SE (D) = 0.26. Using normal theory
the 95 per cent confidence limits of D became UL(D) = 1.30 and LL(D) = 0.26.
From these the 95 per cent confidence limits of P were calculated as UL(P)
= 79.4 units/ml and LL(P) = 39.0 units/ml.

V. DISCUSSION

Anthrax toxins are composed of at least three Factors, I, II, and III by the classification of Stanley and Smith[18] or, respectively, edema factor, protective antigen, and lethal factor according to Beall et al.[11] Insofar as is known, both in vitro-produced toxins, as those used in this report, or in vivo toxins as reported by Klein et al[17] may be quantitated accurately. The procedure further provides an effective reference for quantitating natural resistance or relative immunity as described by Klein et al[17] because the absolute dose of toxins required to elicit a given response will bear a definite relationship to host resistance or susceptibility.

The biological activities of these compounds are numerous and, likely, some responses are still to be discovered. The problem of evaluating activity and mode of action of compounds which have a synergistic biological action is more difficult than for "single compounds." Quantitation, therefore, is important to allow the work of various investigators to be related more exactly to each other. The Fischer 344 rats are commercially available and reference anthrax toxins will be furnished responsible investigators who desire to work with this material for use in establishing units. The methods used in this standardization of these toxins may be appropriate to the standardization of other biologically active toxins.

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